Determination of Pesticide Residues in Honeybees by GC-MS and LC-MS/MS

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Abstract
In recent years, a significant decline in populations of honeybees (*Apis mellifera*) has been recorded. GC/MSD and LC–MS/MS were used to determine pesticide residues in honeybee samples. By GC/MSD analysing six samples of dead honeybees the acetochlor, metolachlor, propiconazole and difenoconazol were detected. By LC-MS/MS analysing thiamethoxam and acetamiprid were detected.

Introduction
In recent years, a significant decline in populations of honeybees (*Apis mellifera*) has been recorded. Through his abuse of the natural environment a man has continually caused damage to the ecosystem and, among other things, has brought about the decrease in the number of bee colonies. Honeybees can be used as the indicators of environmental pollution because of their morphological characteristics and the intense foraging activity, and their ability to retain and bioaccumulate in their bodies substances which they are in close contact with during pollination [1]. In the countries that have a long history of using pesticides in agriculture, such as Serbia [2], one can point to these agrochemicals as one of the important factors underlying wild bee and honey bee colony losses [3]. Growing concern about the impact of pesticides on pollinators is reflected in the enormous literature on the topic in the past few years [4]. The literature of the subject concludes that the presence of pesticides in pollen, honey, wax and other matrices in beehives presents a risk totally different from the effect coused by spraying with plant protection products [3].
According to the data by the Association of bee-keepers of Vojvodina 3200 hives were destroyed in Vojvodina from 2007 to 2012 due to the application of pesticides. The losses of bee colonies are of alarming proportion not only for bee-keepers, honey quality and honey consumers but for agricultural production and the market itself as well [5-7].
The analytical determination of pesticides, although by research groups considered a routine procedure, still constitutes a major challenge especially due to the increasing demand for low limits of detection (LODs) and the complexity of the matrices. The requirement for low LODs is linked to bee toxicity since the honeybee oral LD_{50} and contact LD_{50} are in the ng/g scale for many pesticides. Thus liquid chromatography tandem mass spectrometry (LC–MS/MS) has been used by various researchers [8].
In this article, we describe the evaluation and adaptation of the QuEChERS approach in combination with GC/MSD and LC–MS/MS used to determine pesticide residues in honeybee samples.

Experimental

Chemicals and apparatus. All solvents were of HPLC grade and were obtained from Merck (Darmstadt, Germany). The certified pesticide analytical standards were purchased from Sigma-Aldrich and Dr. Ehrenstorfer (Augsburg, Germany).

For LC analysis, an Agilent 1200 (Agilent Technologies, USA) HPLC system with a binary pump was used. Chromatography separation was achieved using Zorbax C18, 50x4.6 mm, 1.8 µm analytical column from Agilent at a flow rate of 0.4 ml/min with mobile phase consisting of water/methanol with 0.1% formic acid in gradient mode. For the mass spectrometric analysis, an Agilent 6460 Triple-Quad LC/MS system was applied. Agilent MassHunter version B.04.00 software was used for the data acquisition and processing. The analysis was performed in the positive ion mode. The multi source values were as follows: drying gas (nitrogen) temperature 300 °C, drying gas flow rate 5 L/min, nebulizer pressure 40 psi and capillary voltage 3000 V. The detection was performed using the multiple reactions monitoring mode (MRM).

For GC analysis, the Hewlet Packard GC System model 6890, auto sampler Agilent 6890 series injector. The Mass spectrometar Hewlet Packard 5973. GC capillary column: HP5MS (30 m x 0.25 mm x 0.25 µm (5%-Phenyl)-methylpolysiloxane)). Carrier gas: helium, constant pressure 21.82 psi (RTL Pestf-PTV method). GC temparature program: 2 min -70 ºC, 25 ºC/min to 150 ºC (0 min), 3 ºC/min to 200 ºC (0 min), 8 ºC/min to 280 ºC (10 min). Stop time was 41.87 min. The injection volume was 5 µL (PTV, solvent vent mode). PTV temparature program: 0.04 min on 70 ºC, 10 ºC/sec to 280 ºC (10 min), 250 ºC (10 min). Vent flow: 50 mL/min. Vent press: 0.00 psi hold 0.04 min. Purge flow: 60 mL/min start on 2 min. Gas saver: Off. Acquisition mode: SCAN, type of ionisation: EI. Temperature of transfer line was 280 ºC. Temperature MS quadropole of 150 ºC, with the ion source temperature of 230 ºC.

Validation parameters. The validated QuEChERS method according to SANCO/12571/2013 was used for the pesticide residues detections in honeybees. The LOD was calculated by MassHunter Qualitative Software. The linearity was checked using matrix matched standards (MMS) at the concentrations of 10.0-200.0 ng/mL for GC/MSD and 1.0-20.0 ng/mL for LC-MS/MS with the R^2>0.99 for all investigated pesticides. The recovery for the final mass concentration of 0.01 and 0.002 mg/kg was in the range from 89.7-127.4±5-14.8% (for LC-MS/MS) with the addition of the internal standard acetamiprid-D5.

Pesticide extraction

2 g sample +10 mL d. H_2O + 10mLMeCN + 100 µL ISTD (10 µg/mL Acetamiprid-D5)

↓ Shake vigorously for 1 min

Add 4g $MgSO_4$,1g NaCl, 1g Na_3Citrate dihydrate, 0.5g Na_2HCitrat sesquihydrate Shake tube immediately for 1 min

↓ Centrifuge for 5 min at 3500 rpm

Transfer 8 ml of the extract into a PP tube and stor 1 h in the freezer
Transfer 5 ml of the extract into a PP tube containing $MgSO_4$, PSA, C18; Shake for 30 s

↓ Centrifuge for 5 min at 3500 rpm

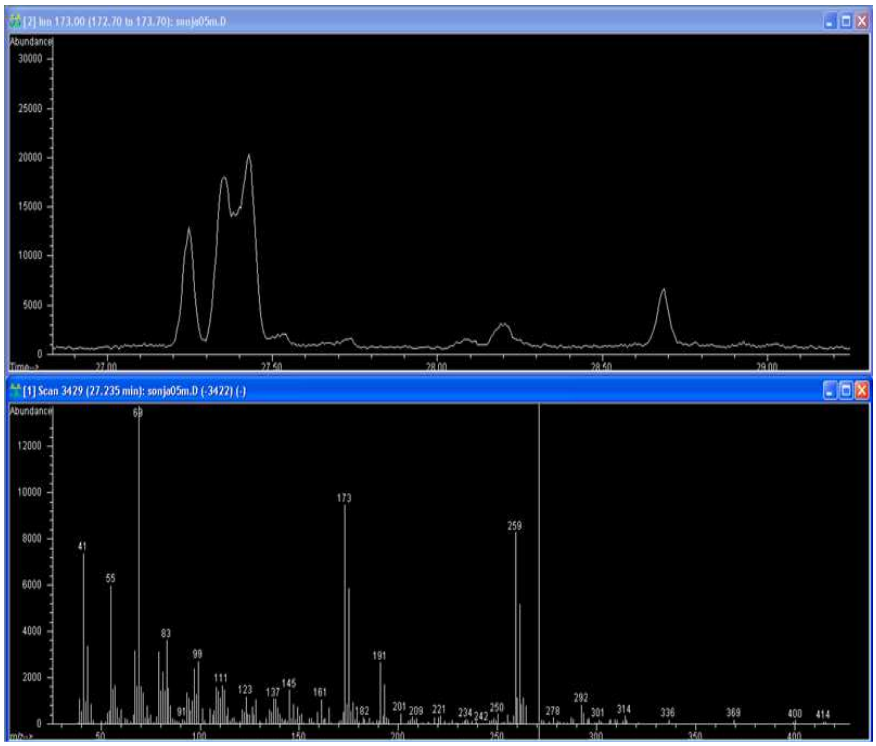
Transfer 200 µL into a vial, evaporate to dryness ↓ Reconstitute in 200 µL of mobile phase then LC-MS/MS	Transfer 2 mL into a vial, evaporate to dryness ↓ Reconstitute in 1 mL of hexan/aceton then GC-MS

Results and discussion

The analysis comprised the detection of pesticide residues in six honeybees samples collected from the localities of Čerević and Radojevo. The validated QuEChERS method according to

SANCO/12571/2013 was used for the pesticide residues detections in honeybees by LC-MS/MS and GC-MS.

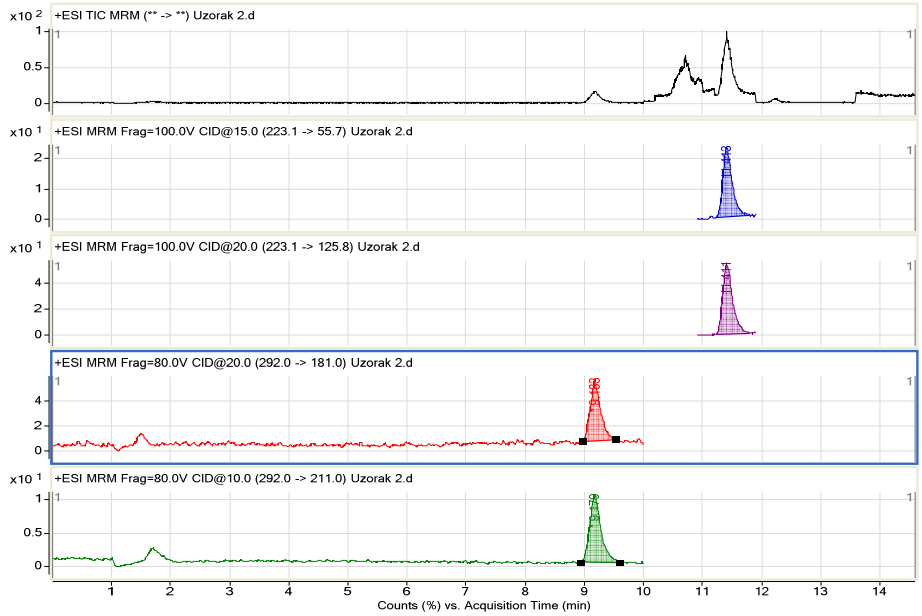
Figure 1. Detected pesticides in a honeybees sample obtained by GC-MS



By GC/MSD analysing six samples of dead honeybees the acetochlor, metolachlor, propiconazole and difenoconazol were detected. The detections of acetochlor were in the range from 12.8 to 18.5 ng/g, for metolachlor 49-72 ng/g, for propiconazole 19 to 29 ng/g, and for difenoconazol from 390 to 420 ng/g. Acute oral toxicity expressed as LD_{50} for acetochlor and propiconazol is >100 µg/bee, while the acute contact LD_{50} is >200 µg/bee while for difenoconazol acute oral is >100, and the acute contact is >187 µg/bee. According to EPA, metolachlor is not bee toxic [3].

By LC-MS/MS analysing thiamethoxam and acetamiprid were detected. In one sample thiamethoxam was found at the concentracion level of 18 µg/kg, and acetamiprid was found in three samples between 0.012 and 0.033 mg/kg. Acute oral toxicity expressed as LD_{50} for acetamirid is 14.53 µg/bee, while the acute contact LD_{50} is 8.09 µg/bee; for thiamethoxam acute oral is 0.005, while the acute contact is 0.024 µg/bee.

Figure 2. Detected pesticides in a honeybees sample obtained by LC-MS/MS



A typical risk assessment considers only the acute toxicity of pesticides by contact or oral exposure in 24/48 hours, thus ignoring the negative effects derived from the constant exposure to pesticide residues over longer periods [3].

Conclusion

Taking into consideration the results GC-MS and LC-MS/MS analyses of honeybees samples, there is a significant and justified doubt that the detected pesticides brought about the death of honeybees.

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